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(54) Title: CRYOPRESERVATION OF PORCINE EMBRYOS

(57) Abstract

Porcine embryos are cryopreserved using a method for reducing the level of lipid within cells of the embryo followed by freezing the embryo. Reduction of the level of lipid can be achieved either by polarizing the lipid within the embryo by centrifugation of the embryo, followed by rapid freezing or by microsurgical removal of the polarized lipid. Alternatively the level of lipid may be reduced by culturing the embryo in vitro. The reduction of the level of lipid before freezing results in good survival rates and can be used on zona intact porcine embryos.

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CRYOPRESERVATION OF PORCINE EMBRYOS

FIELD OF THE INVENTION

This invention relates to the cryopreservation of porcine embryos.

BACKGROUND OF THE INVENTION

The introduction of new genetic material into an animal breeding facility such as a piggery is an important and continuing activity to minimise the negative impact of inbreeding. Additionally, even in outbred herds, it is advantageous to introduce new commercially beneficial genetic traits, such as disease resistance or superior growth characteristics.

In the past the introduction of new genetic material has involved the introduction of new animals into a herd. The logistics of transporting live animals is commercially unwieldy, and all the more so because of the major concerns that are held about disease transmission. Such concerns about disease have lead to the introduction of barriers by some countries to the importation of live animals.

Presently new genetic stock is normally introduced into pig herds by importing semen with which sows are artificially inseminated. The drawback of introducing genetic material using only semen is that backcrosses over several generations are required to achieve the desired genotype.

An alternative approach that is sometime used involves the hysterectomy of term pregnant females and introduction of those offspring to surrogate sows. This technique minimises the risk of transfer of infectious agents between breeding facilities, however, the technique is very limited because the progeny can only be transported for short distances.

There have been great advances in the cryopreservation and implantation of embryos in species other than pigs over the past 2 decades, particularly with cattle. As a result of these advances, cryopreserved bovine embryos are now used extensively for transport of genetic material. In contrast to semen, embryos are preferable for the conservation or introduction of new genetic material.

The advances in cattle have not been mirrored in some species of animal of which pigs are an example and there are still several problems associated with freezing pig

embryos. These problems are not specific for freezing embryos and are also applicable to oocytes.

One of the major problems of freezing embryos is that damage to cells is occasioned as a result of ice crystal formation. Cryoprotectants have been used in conjunction with embryos for several species to minimize the impact of ice crystal formation. Two general types of cryoprotectants have been used, permeating substances and non permeating substances. Permeating cryoprotectants enter cells and include substances, such a DMSO, or glycerol and act to effectively lower the freezing point. These types of cryoprotectants are used to replace intracellular water reducing the likelihood of lethal intracellular ice crystal formation. Non-permeating cryoprotectants do not enter the cell. The presence of cryoprotectant outside of a cell can cause the cells to dehydrate, removing intracellular water preventing the formation of large ice crystals within the cell, which would otherwise be lethal to cells.

Another effect of freezing is on the membranes that form part of the cellular structure; either as the external membrane that envelope cells to ensure integrity of the cells or internal membrane structures such as those forming the endoplasmic reticulum. These membranes comprise a bilayer of phospholipid molecules with other structural components, including protein components embedded within the bilayer. The phospholipids usually exist in a fluid state, the fluid state being essential for the proper functioning of the membrane. If the temperature of the membrane is allowed to drop to somewhere between 15 to 20°C a phase transition occurs where the lipids in the membrane are no longer in a fluid state and leads to a loss of viability (Petit and Edidin, Science **184**; 1183 (1974)), and the stage at which this loss of viability happens is highly dependent on its lipid composition (Quinn in Quinn and Cherry (eds) "Structural and dynamic properties of lipids and membranes". Portland Press Ltd., London, UK, 1992 pp29).

A number of different freezing and thawing regimes and techniques have been developed for bovine and other embryos. A review of this general area has been written by H Niemann in Theriogenology (1991) Vol 35 page 109. These types of techniques are not universally applicable to all animals and variations exist even within the same species.

There are essentially three basic approaches that are used to freeze embryos, a) controlled freezing and thawing; where a gradual reduction or increase in temperature is

used b) vitrification; using high concentrations of cryopreservant, and c) Ultrarapid freezing; using very rapid freezing techniques.

The reason for the difficulty in freezing porcine embryos is not at all clear. A brief explanation of the development of the embryo development will assist in understanding the problem.

Porcine embryos (as do other embryos) develop from a single cell (fertilised egg), which divides during the early stages of cell development into a ball of cells without ever increasing its diameter. From the single cell to the blastocyst stage the embryo is covered by a protective layer known as a zona pellucida. The ball of cells in the early stages (from about the 16 cell stage) is referred to as a morula. The protective layer is thought to protect the embryo against a variety of agents, including physical damage, and infectious agents, such as viruses, from penetrating into the cell mass of the embryo.

The porcine embryo develops from the morula to the blastocyst stage on day 4 to 5. The blastocyst consists of a single peripheral layer of cells, the trophectoderm or trophoblast, and a group of cells inside the blastocoel cavity termed the inner cell mass cells from which the foetus develops.

At about day 5 to 6 in the pig (the onset of estrus = day 0) the blastocyst begins to increase its mass, and this stage is known as an expanded blastocyst. The expanded blastocyst is still enveloped by the zona pellucida. The zona pellucida thins as a result of enzymic digestion at about day 6 to 7 and the blastocyst "hatches" out from the zona pellucida. Once fully hatched the blastocyst can become implanted and develop further, and at this stage is referred to as a hatched blastocyst. The hatching and fully hatched blastocyst is far more susceptible to physical damage and to infection. The term peri-hatching or peri-hatched has been coined to refer to embryos that are at any stage of development between and including expanded to fully hatched blastocysts.

There have been several reports on the survival of porcine embryos after cooling or freezing. Nagashima *et al* (Jpn. J Anim. Reprod. 341 123 (1988) assessed embryo survival by *in vitro* culture following exposure to 6°C for 1 or 16 hours and concluded that embryos at the expanded and hatched blastocyst stage survive cooling compared with early blastocysts or morulae that do not. Blastocysts hatched *in vitro* were shown to be able to maintain their viability and develop to normal foetus after exposure to 11°C. The freezability of the peri-hatched staged blastocysts was further confirmed by

freezing experiments to -20°C (Nagashima et al Jpn. J Anim Reprod. 35 ; 130 (1989)). Hayashi et al. (Vet Rec, 125; 43 (1989)) succeeded in obtaining 5 piglets from 11 expanded blastocysts which had been briefly frozen at -35°C with 1.5 M glycerol.

The freezability of the peri-hatching stage blastocysts to allow storage in liquid nitrogen has subsequently been demonstrated by three groups. Kashiwazaki *et al* (Therigenology 35;221 (1991) and Vet Rec 16;256 (1991)) used *in vitro* hatched blastocysts which had been collected from Large White and cross bred (Meishan x Meishan/Large White F_1) donors and cultured for 18-24 hours in modified KRB (Davis *et al* J. Anim. Sci. 46;1043 (1978)) supplemented with 4mg/ml BSA and 10% foetal calf serum (FCS). These embryos were frozen by a conventional slow cooling method in the presence of 1.5M glycerol to -35°C (seeded at -6.8°C) followed by plunging into liquid nitrogen. After thawing in a 35°C water bath, the cryoprotectant was removed by step-wise dilution. Thirty two embryos were transferred to a day-6 recipient and three Large White and one crossbred piglets were born.

A live piglet was also produced by transferring 8 Meishan hatched blastocysts (day-7) frozen with 1.4M (10%) glycerol in PBS (Phosphate Buffered Saline) supplemented with 50% FCS and 10% egg yolk by slow cooling and seeding at -7°C with silver iodide (Fujino *et al,* Cryobiology 30; 299 (1993)). These embryos were transferred to a day-5 recipient with 4 unfrozen Large White hatched blastocysts to assist in the maintenance of pregnancy.

Kameyama *et al.* (Proceedings of 78th Annual Meeting of The Japanese Society of Animal Reproduction. Niigata, Japan , 1990, p22 Abstr.) cryopreserved expanded blastocysts collected on day-6 using a freezing solution containing 1.5 M glycerol, 0.05% lecithine and 15% FCS in PBS. The embryos were cooled rapidly from room temperatures to the seeding temperature (-6.8°C) and then cooled slowly to -35°C, followed by plunging into liquid nitrogen. Transfer of the 20 frozen-thawed embryos to a single recipient resulted in the birth of 2 live and 2 stillborn piglets.

In vitro culture of embryos frozen in liquid nitrogen showed that the survival rates were between 17-50% for expanded blastocysts (Hayashi *et al* Vet Rec 125;43 (1989)), Kashiwazaki et al Vet Rec 16; 256 (1991)), Nagashima et al Theriogenology 37;839 (1992), Cameron et al Proceedings of 12th International Pig Veterinary Society. Hague, The Netherlands, 1992 p476 Abstr., Dobrinsky et al Proceedings of the 4th International Conference on Pig Reproduction, Columbia, USA, 1993, p35 Abstr.) and between 38% to 96% for hatching and hatched blastocysts (Fujino et al Cryobiology

30;299 (1993), Hayashi *et al* Vet Rec **125**;43 (1989)), Kashiwazaki et al Vet Rec **16**; 256 (1991)), Nagashima et al Theriogenology **37**;839 (1992), Cameron et al Proceedings of 12th International Pig Veterinary Society. Hague, The Netherlands , 1992 p476 Abstr., Dobrinsky et al Proceedings of the 4th International Conference on Pig Reproduction, Columbia, USA, 1993, p35 Abstr., French et al Proceedings of The 23rd Annual Conference of Australian Society of Reproductive Biology, Sydney, Australia 1991, p119 Abstr.). In contrast, embryos younger than expanded blastocyst stage as well as hatched blastocysts having a diameter larger than 300μm do not survive freezing, indicating that the freezability of porcine blastocysts peaks immediately after hatching and then decreases rapidly.

From these data, it appears that the most appropriate conditions presently known for cryopreservation of porcine embryos are as follows:-

(A) Blastocysts at peri-hatching stages must be chosen. They can be developed from earlier stages using *in vitro* culture with conventional embryo culture media such as Whittingham's M-16 (J. Reprod. Fert **14(suppl.)**7 (1971)). Whitten's (Adv. Biosci. **6**; 129 (1971)) or KRB (Davis and Day J. Anim. Sci. **46**; 1043 (1978)). Addition of BSA to these media appears to be crucial to confer freezability (Nagashima et al Theriogenology **37**; 839 (1992)).

(B) As a cryoprotectant 1.5 M of glycerol seems to be effective, though a thorough screening for other cryoprotectants has yet to be carried out.

(C) Conventional slow cooling methods can be used, probably with wide tolerance for cooling rates as slow as 1°C/min or direct cooling from ambient temperature to seeding temperature (-6 to -7°C), followed by slow cooling at 0.3°C/min to -35 to -38°C and plunging into liquid nitrogen.

(D) After rapid thawing in a water bath at 35-37°C, step-wise dilution of the cryoprotectant using step-down sucrose from 0.3 - 0.5M to 0 M appears to protect the embryonic cells from osmotic shock.

Thus attempts have been made to freeze embryos of pigs at a variety of stages of development, however, only embryos at the perihatching stage have been successfully frozen, and the rate of successful pregnancies following storage of embryos has been very low.

It is however highly desirable to have access to frozen embryos that have not yet "hatched" or whose zona pellucida has not thinned, because where hatching or thinning has occurred such blastocysts are far more susceptible to infectious agents or physical damage.

Several possible explanations exist as to why porcine embryos are more susceptible to damage, following freezing and thawing, than other embryos. Early stage porcine embryos have a large number of lipid droplets present in the cytoplasm of the cells. These lipid droplets can be stained by Sudan III (a lipid specific stain) and by Nile blue sulphate (a stain specific for neutral lipids). As the embryo develops towards the perihatching stage the size of the lipid droplets decreases, as does the total lipid content. It is thought that the lipid droplets act as an energy store for use by the developing embryo.

It has been postulated that this reduction of lipid content is associated with the freezability of embryos because changes in the number and size of these droplets have been correlated with stages at which the embryos from different species can be successfully frozen. Porcine embryos contain a large number of lipid droplets throughout early embryonic development and have not been successfully frozen before the perihatching stage at which time there is a marked reduction in the amount of lipid contained within these vesicles.

The postulated effect of the lipid droplet on the freezability of embryos has been suggested to be either due to an effect that lipid droplets may have on the membrane of the embryo, or alternatively by some physical means, perhaps in preventing the cryoprotectant from penetrating into the embryo, or acting as nuclei for ice crystal formation. Which, if any, of these explanations is correct is however not clear, and before the present invention it was not clear that level of lipid was responsible for the poor survival of embryos after freezing.

The temperature below which porcine embryos at a stage earlier than peri-hatching do not survive is 15°C. This is also approximately the temperature at which lipid phase separations occur in mammalian cells. Lipid phase separations occur in plasma membranes, internal membranes and also the lipid droplets. Structural changes in lipid droplets also occur around this temperature - they coalesce and form larger droplets. Both changes may cause irreversible damage to the embryo at this stage. It has been further suggested that the high lipid content leads to uneven intracellular ice formation which may also be lethal (Niemann et al Theriogenology 1991 35; 109).

SUMMARY OF THE INVENTION

It has been found according to one aspect of this invention that the survival rate of porcine embryos to cooling and/or freezing can be greatly enhanced by reducing the level of lipid derived from lipid droplets in cells of the embryo.

It is found also that the survival of embryos after freezing can be achieved much earlier than at the peri-hatching stage by reducing the level of lipid derived from lipid droplets in cells of the embryo, and as early as the single cell stage of embryo development.

The invention therefore could in a broad form be said to reside in a method of cryopreserving a porcine embryo comprising the step of reducing the level of lipid derived from lipid droplets in cells of the embryo, followed by the step of reducing the temperature of the embryo to a storage temperature suitable for storage of the embryo whilst the reduced level of lipid droplets is maintained, the level to which lipid is reduced such that significant damage is not sustained by said embryo when the step of reducing the temperature is performed.

The extent to which lipid derived from lipid droplets needs to be removed in order that an improvement in the viability of cryopreserved embryos is obtained is not fully defined. The experiments conducted thus far show that if approximately half of the observable lipid is allowed to remain in cells of the embryo then minimal benefits are obtained. Photomicrographs of embryos, where as much lipid has been removed as is technically possible, indicate clearly that some lipid remains. Therefore benefit is obtained without total removal of lipid derived from the lipid droplets. The cut-off point at which benefits are derived has thus not been quantitatively defined, and from a point of view of practicing the invention lipid level may be a parameter that might be varied to best suit the technical means of reducing the lipid level within cells of the embryo. It would seem however that removal of substantially all of the visible lipid as is possible by any appropriate technical means is desirable.

In an alternative form, the invention could be said to reside in a method of cryopreservation of a porcine embryo, comprising the steps of removing substantially all visible lipid derived from droplets in cells of the embryo and the step of reducing the temperature of the embryo to a temperature suitable for the storage of the embryo.

The present invention has particular benefit in that it permits cryopreservation of porcine embryos at a stage of development earlier than the perihatching stage.

It is to be understood that the temperature at which embryos are to be stored is most desirably that of liquid nitrogen. Alternatively where liquid nitrogen facilities are not available then the temperature attainable by any freezer that might be suitable for the purpose.

The lipid derived from lipid droplets might conveniently be removed from cells by centrifuging the embryo so as to polarize the lipid in the perivitelline space, between the cells of the embryo and the zona pellucida.

After polarization (or removal of lipids from cells), the lipid may be physically removed from the embryo. This physical removal can be achieved by piercing the zona pellucida of the embryo with a micropipette, contacting the lipid with the micropipette and applying suction to the lipid to remove lipid through the micropipette. The micromanipulation is done with the assistance of a micromanipulator and a microscope.

During the physical removal of lipid derived from lipid droplets of the embryo, the embryo is preferably treated with a substance such as Cytochalasin C or Demecolcine (which are termed cytoskeletal inhibitors) which relax the cytoskeletal elements to thereby minimise damage caused by piercing the cells.

The lipid droplets may be removed at the single cells stage of the embryo. This facilitates the removal of the lipid from the cytoplasm. It is found that cells still survive the physical removal of lipid droplets and can be frozen successfully immediately after such treatment.

The cells of the embryo may also be cultured after removal of lipid before temperature is reduced to the storage temperature. Thus cells may be cultured for 14 to 18 hours before being cooled. Where lipid has been removed from single cells, such embryos are usually at the 2 or 4 cell stage. This extra treatment is suggested to further enhance the survival rate of embryos. The reasons for this enhanced survival may be that the embryo has had time to recover from the trauma of lipid removal. Additionally further lipid removal may be achieved, because the lipid remaining after removal is further depleted because of the energy demand of the developing embryo.

There are drawbacks associated with the physical removal of lipid droplets from embryos. One difficulty is that the described method preferably involves microsurgical techniques, which are complicated and time consuming. Where the technique is to be

used on a large scale the associated equipment, labour and time costs are likely to be commercially prohibitive.

A further drawback is that microsurgical techniques may and can be destructive to embryonic cells. Such techniques can also damage the zona pellucida, a membranous structure protecting embryos from infection by pathogens. Accordingly viability of frozen embryos so treated is likely to be lower than might be desirable.

For these reasons a protocol for cryopreserving porcine embryos using a non-invasive method for delipation is desirable.

Earlier experiments by the applicant have shown that polarisation of cytoplasmic lipid in embryos by centrifugation did not confer freezability on embryos. This is because the polarised lipid left in the peri vitelline space is redistributed into the cytoplasm before the embryo freezes using conventional slow cooling methods. However, it is proposed that if the embryos with polarised lipid are frozen rapidly using rapid freezing or vitrification methods for example, there is insufficient time for the lipid to return to the cytoplasm and hence the embryos can be successfully cryopreserved.

In a further form of the present invention the lipid droplets are polarised away from the cells of the embryo and the embryo is frozen rapidly so that the lipid droplets are not able to redistribute back into the cells to a significant extent before freezing.

A convenient method for polarising the lipid within the embryo is by centrifugation at a suitable speed, so that the lipid droplets are polarised to the peri-vitelline space, and the temperature is reduced at a rate sufficient to pre-empt the redistribution of lipid back into the cytoplasm of the cells.

It is to be understood that this method of cryopreservation may be part of a broader embryo transfer or breeding program and that the invention also resides in an embryo that has been cryopreserved by any of the methods outlined above. It is to be understood that the invention also encompasses animals produced from embryos that have been so cryopreserved.

An alternative method for depleting lipids from the embryos may be to collect embryos from sows at or close to ovulation, and culture such embryos. This approach is suggested as a possible means to increase the rate of lipid depletion, because culture of embryos is less efficient than development within a sow, and consequently more energy

depleting. It may be possible to deplete the level of lipid droplets within an embryo by *in vitro* culture techniques without polarisation.

Any one of a number of cryoprotectants may be used and the regime and rate at which the temperature is reduced can also be optimised.

It is to be understood that whilst it is found highly desirable to use this invention for porcine embryo cryopreservation, it may also be desired to use the present method for the cryopreservation of embryos derived from other species.

In a further aspect the invention could be said to reside in an embryo which is cryopreserved using the method of cryopreservation of this invention.

In another aspect the invention could be said to reside in a method of implantation of an embryo, comprising the step of cryopreserving a porcine embryo as described or defined in this specification, thawing the embryo and implanting the embryo into a recipient.

The invention is also to be understood to include the method of producing an animal using cryopreserved embryos of this invention, which embryos are then thawed and implanted into recipients and allowed to gestate to give rise to said animal. The invention is also to be understood to encompass an animal produced by such method.

EXAMPLES

For a better understanding the invention will now be described with reference to the examples.

Throughout this specification the term delipation is used, wherein the term delipidization has been used elsewhere the two terms are to be understood to have the same meaning. The term delipation is to be understood not to mean total removal of lipid droplets within the cytoplasm of the embryos but rather to mean a reduction of the lipid, where the majority of lipid droplets are no longer apparent within the cytoplasm of embyronic cells.

Many of the techniques used in the examples are ones that are practiced by skilled embryologists. A general reference to typical methods that might be employed may be found in the reference "Embryo Transfer in Large Domestic Mammals" by Murray F.

A., in "Methods in Mammalian Reproduction" edited by J.C. Daniel Jr (Academic Press 1978).

EXAMPLE I

Porcine 1 cell stage embryos treated with 7.5µg/µml cytochalashin B were centrifuged to polarize the lipid droplets in the cytoplasm. The resultant lipid layer was then removed by micromanipulation using a bevelled micropipette. Sham operated embryos were treated with cytochalasin B and centrifuged but not delipated. The delipated embryos together with the sham operated and intact controls were then exposed to 4°C for 1 hr in PBS+10%FCS either within 2 h or after a 14 to 18h period of culture in Whitten's medium + 1.5% BSA. Both groups of embryos and the controls were subjected to *in vitro* culture to compare their survival. Embryos wre deemed to have survived if they cleaved following thawing

It is known that the majority of damage to cells in pig embryos is at temperatures at between 15 - 20°C, and the survival of embryos at the temperatures described in this example are expected to reflect the survival of embryos when frozen.

TABLE 1. Survival of porcine delipated embryos after cooling to 4°C.

Treatment	No. embryos cooled	No. survived	Most advanced development
Delipated and cooled immediately	22	14 (63%)	8-cell
Sham operated cooled immediately	14	1 (7%)	2-cell
Control cooled immediately	14	0 (0%)	
Delipated and cooled at 2-4 cell	24	22 (92%)	Blastocyst
Sham operated cooled at 2-4 cell	15	4 (27%)	cleaved once
Control cooled at 2-4 cell	11	0 (0%)	

As shown in Table 1, most of the delipated embryos cleaved after cooling with development of the morula-blastocyst stage, whereas all of the control embryos lysed within 24 hours. The sham operated embryos appeared more tolerant to cooling but none cleaved more than once. In vitro culture for 14 to 18 hours of delipated embryos enhances survival.

EXAMPLE 2.

Collection of Embryos

A total of 45 pregnant crossbred gilts (Large White x Landrace) were aborted by intramuscular (i.m.) injection of 1 mg of the prostaglandin $F_{2\alpha}$ analogue cloprostenol (Estrumate; Pitman-Moore, New South Wales, Australia) between 24 and 40 days after mating, followed by a second injection of 0.5mg cloprostenol 24 h later. At the time of the second injection, 1000 IU of eCG (Pregnecol; Heriot AgVet, Vic, Australia) was administered i.m. Ovulation was induced by i.m. injection of 500 IU hCG (Chorulon; Intervet, New South Wales, Australia) administered approximately 72 h after eCG. On the next afternoon (Day 0), the gilts were mated with fertile Large White boars, and zygotes were surgically collected 49-53 h after the hCG injection (Day 1). Embryos collected from each donor were allocated across all treatments in each experiment.

Removal of Lipid Droplets from Embryos.

Embryos were centrifuged in modified PBS (PB1) (Quinn et al J Reprod. Fertil 1982; 66:161-168) containing 10% foetal calf serum (FCS) and 7.5 µg/ml cytochalasin B (Sigma Chemical Co., St Louis, MO) in 1.5 ml micro-centrifuge tubes (Treff Lab, Switzerland) at 12000 xg for 8 min at room temperature in order to polarize the lipid droplets in the cytoplasm. The resulting lipid layer was then removed as much as possible (fully delipated) or approximately half was removed (partially delipated),by micromanipulation using a bevelled suction pipette (30µm in diameter) attached to Narishige micromanipulators (MO-108l Tokyo, Japan) under a Nikon inverted microscope (TMDl Tokyo, Japan). Embryos were held in the same medium for micromanipulation as for centrifugation. Sham-operated embryos were treated with cytochalasin B and centrifuged, but were not penetrated with the suction pipette or delipated.

After lipid removal or sham operation, the embryos were washed and maintained in PB1 +10% FCS at 37° C until chilling or culture.

Culture and Chilling of the Lipid-Removed Embryos

Embryos were chilled within 2 h after lipid removal or sham operation or were maintained in culture for 14-18 h and then chilled. Embryos were cultured in 50-µl droplets of Whitten's medium (Whitten Adv Biosci 1971; 6:129-141) supplemented with 15mg/ml BSA (fraction V; Pentex, Miles IL) under paraffin oil in a plastic petri dish held under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in humidified air at 38.6°C.

For chilling, embryos were loaded into 0.25 ml plastic straws with PB1 + 10% FCS; to expose the embryos to 4°C, the straws were submerged in iced water for 1 hr and then rewarmed to 37°C by being laid on a warming tray. The recovered embryos were then cultured in Whitten's medium as described above for assessment of their survival. Embryos that cleaved at least once were assessed as surviving. The 1-cell and 2- to 4-cell delipated embryos were cultured for 144 and 120 h, respectively. Some of the cultured embryos (n=20-45) were fixed and stained by aceto-alcohol and 1% aceto-orcein in order to count cell numbers.

Statistics

Survival rates and developmental rates of embryos were compared using ANOVA. When ANOVA revealed a significant difference between groups, the difference was analysed by X^2 test. Student's *t*- test was used to compare mean cell numbers of embryos.

Experiment 1 Chilling of lipid-removed embryos at the 1 cell or 2- to 4-cell stages

Chilling sensitivity of delipated, sham-operated, and intact embryos (control) was investigated. These embryos were chilled at the 1-cell stage or at the 2- to 4- cell stage following culture, and their survival (cleavage and subsequent division) was assessed by culture for 144 h and 120 h, respectively.

A total of 120 zygotes were fully delipated. Ninety four (78.3%) embryos in which the cytoplasm remained intact after micromanipulation were chilled either within 2 h of lipid removal while still at the 1-cell stage or after further development in culture to the 2- to 4-cell stage. Control and sham operated embryos at identical stage of development were chilled concurrently.

As shown in Table 2, more than 60% of the delipated embryos cleaved after chilling with development beyond the 8 cell stage, whereas all of the control embryos had lysed within 24 h. Although overall survival rates of the delipated embryos were almost

equal between those chilled at the 1 cell stage and those chilled at the 2- to 4- cell stage, more embryos developed beyond the 8-cell stage when they were chilled at the 2- 4- cell stage (44.4% vs 18.4%, $p < 0.01$). This included 5 (11.1%) embryos that developed to the blastocyst stage. None of the sham-operated embryos cleaved more than once after chilling at the 1-cell stage or developed beyond the 5-cell stage after chilling at the 2- to 4- cell stage.

TABLE 2. Survival of the lipid-removed (delipated) porcine embryos after chilling to 4°C at the 1-cell and 2- to 4- cell stage.

Embryos	Embryonic stage at chilling *	No. of embryos Chilled**	No. of Embryos surviving (%)	No. of embryos developing to ≥ 8-cell stage (%)
Control	1-cell	19	0 (0)	0 (0)
Control	2- to 4- cell	16	0 (0)	0 (0)
Sham-operated	1-cell	19	1 (5.3)[a]	0 (0)
Sham-operated	2- to 4- cell	18	4 (22.2)[ab]	0 (0)
Delipated	1 - cell	49	32 (65.3)[c]	9 (18.4)[d]
Delipated	2- to 4- cell	45	28 (62.2)[c]	20 (44.4)[d]

[a,b,c] Values with different superscript within column differ significantly, [a] vs [c] $p<0.001$; [b] vs [c] $p<0.005$; [d] $p < 0.01$

* Embryos delipated at the 1-cell stage were chilled immediately or cultured 14 - 18h before being chilled at ~4 cell stage.

** Three experiments were repeated in each group

Experiment 2: Comparison of chilling sensitivity after partial and full lipid removal. The aim of the second experiment was to investigate whether partial removal of lipid could render porcine embryos equally tolerant to chilling without compromising their *in vitro* developmental ability. Fully and partially delipated embryos were chilled at the 2- to 4- cell stage and their development was examined by culture for 120 h.

As shown in Table 3, the number of partially delipated embryos that survived chilling was similar to the number for fully delipated embryos (77.5% vs 90.4%, no significant difference); however, fewer developed beyond the 8-cell stage (32.5% vs. 73.1%, $p<0.001$) or to the blastocyst stage (7.5% vs 26.9%, $p<0.05$).

TABLE 3. Comparison of the *in vitro* development of the fully and partially lipi-removed (delipated) porcine embryos after chilling

Embryos	No. of embryos Chilled	No. of embryos surviving (%)	No. of embryos developing to: ≥ 8 - cell stage (%)	≥ blastocyst stage (%)	Mean (±SE) number of cells **
Fully delipated	52*	47 (90.4)[a]	38 (73.1)[b]	14 (26.9)[d]	12.8± 5.2[g]
Partially delipated	40*	31 (77.5)[a]	13 (32.5)[c]	3 (7.5)[e]	11.9±9.2[g]

[a] No significantly different
[b,c] $p<0.001$; [d,e] $p< 0.05$
* Six experiments were repeated in each group.
** Twenty embryos in each group were used to count cells.

Experiment 3 : The ability of delipated embryos to develop in vitro.

The developmental ability in vitro, without chilling, of fully and partially delipated embryos was compared with that of intact (control) zygotes. All embryos were cultured from the 1-cell stage for 144 h.

Table 4 represents the comparison between the development of fully and partially delipated embryos and that of control zygotes after culture for 144 h. No significant difference was seen in development between partially and fully delipated embryos. Moreover, the developmental ability of the delipated embryos appeared to be comparable to that of the control zygotes, except that fewer of the partially delipated embryos developed to the blastocyst stage (25% vs 57%; $p < 0.05$; Table 3). The delipated embryos cleaved and developed at almost the same rate as the controls. The mean cell numbers of the delipated embryos tended to be lower than those for control embryos, but this difference was not significant. Cell numbers in blastocysts derived from fully delipated embryos ranged from 16 to 68; this was comparable with the range for the control blastocysts of 23 to 75.

FIG. 1. is a comparison of the *in vitro* development of porcine embryos from which lipid was partially and fully removed (delipated). Note that polarized lipid droplets left in the partially delipated embryo (a-d) have redistributed throughout the cytoplasm during culture and the blastomeres of the partially delipated embryos are darker in color than those of the fully delipated embryos (e-h). Photographs were taken immediately

after lipd removal (a and e) and at 48 h (b and f), 72 h (c and g) and 120 h (d and h) in culture. Magnification is x200.

As shown in Figure 1, the cytoplasm of partially delipated embryos was darker in color than that of fully delipated embryos throughout all stages of their development in vitro, indicating that the remaining lipid droplets had redistributed throughout the cytoplasm.

TABLE 4. *In vitro* developmental ability of lipid-removed (delipated) porcine embryos.

Embryos	No. of Embryos cultured	No embryos developed to: Cleaved (%)	≥ 4- Cell (%)	≥ 8-cell (%)	Blastocyst (%)	Mean (±SE) number of cells**
Control	30	26 (86.6)[a]	25 (83.3)[a]	24 (80.0)[a]	17 (56.6)[b]	34.0 ±18.3[g]
Partially delipated embryos	36	29 (80.6)[a]	29 (80.6)[a]	26 (72.2)[a]	9 (25.0)[c]	18.5±15.0[a]
Fully delipated embryos	48	45 (93.8)[a]	45 (93.8)[a]	38 (79.2)[a]	19 (39.6)[bc]	19.5 ± 14.6[a]

Values with different superscipt within columns differ significantly.
[b,c] ($p<0.005$).
* Three experiments were repeated in each group
** Twenty five to 45 embryos were used to count cell numbers.

EXAMPLE 3

The methods employed here are similar to those employed in the previous examples. 158 delipated 2-4 cell embryos were frozen and stored in liquid nitrogen. These were thawed, cultured for three hours and their morphology assessed at the end of this period Embryos having the same morphology before freezing were judged to be suitable for transfer (normally color of the cytoplasm and integrity of the cytoplasmic membrane can be the criteria). 95% of embryos showed little or no evidence of damage and were transferred to 4 recipients (30-40 embryos per recipient). Three of the four recipients were confirmed to be pregnant at 50 days of gestation using ultrasound to detect foetuses. Skeleton, membranes, internal organs and heart movement were visualised.

EXAMPLE 4

This example of the invention is proposed to comprise three steps, including centrifuging embryos to polarise cytoplasmic lipid, equilibrating the centrifuged embryos with cryoprotectant and freeze thawing embryos.

The embryos can be centrifuged at 1-cell to blastocyst stage approximately 12800 x g for 10 to 15 min to polarize cytoplasmic lipid. Embryos can be centrifuged with or without cytoskeleton inhibitor (for example 5-10μg/ml cytochalasin B, D). After centrifugation the lipid can visualised microscopically as polarised in the peri vitelline space away from the blastinere and inside the zona pellucida.

After centrifugation the embryos are equilibrated with cryoprotectant for 1 to 20 min. Examples of cryprotectants that might be used include, 1-7 M DMSO, glycerol, ethylene glycol, propandiol with or without sucrose, toleharose or other sugars that are commonly used. To minimise the time for lipid to re-enter the cytoplasm, embryos can be equilibrated with cryoprotectant during the centrifugation.

The embryos equilibrated with cryoprotectant are loaded into a 0.25ml plastic straw with the freezing solution and cooled directly to -30 to -150°C, followed by plunging into liquid nitrogen.

Embryos are thawed in a water bath at 10 to 37°C. Cryoprotectant is removed from embryos by stepwise dilution followed by thorough washing with culture medium.

EXAMPLE 5

Embryos at the 2 to 4 cell stage were delipated, by use of a micropipette after polarizing the lipid, as described earlier. Delipated embryos were cultured for approximately 16 h and 33 were transferred each to two recipients both of whom tested pregnant at day 40 by ultrasound. Both pigs farrowed and produced normal sized litters of 8 and 10 piglets respectively. Table 5 shows the results for each of the recipients separately and together.

TABLE 5. Delipated porcine embryos are able to develop to term

Embryos*	Recipients	Pregnancy	Piglets (%)
n=33	1	+	10 live 1 stillborn (33%)
n=33	1	+	8 live 2 stillborn (30%)
Total n=66	2	2/2 (100%)	22/66 (32%)

* Delipated 2-4 cell stage/developed to 4 to 8 cell stage in culture for approximately 16 h.

This finding demonstrates that the removal of lipd from the embryo does not affect its ability to develop to term.

CLAIMS

1. A method of cryopreserving a porcine embryo comprising the step of reducing the level of lipid derived from lipid droplets in cells of the embryo, followed by the step of reducing the temperature of the embryo to a storage temperature suitable for storage of the embryo whilst the reduced level of lipid droplets is maintained, the level to which lipid is reduced such that significant damage is not sustained by said embryo when the step of reducing the temperature is performed.

2. A method of cryopreservation of a porcine embryo, comprising the steps of removing substantially all visible lipid derived from droplets in cells of the embryo and the step of reducing the temperature of the embryo to a storage temperature suitable for storage of the embryo.

3. A method for cryopreservation of a porcine embryo comprising the step of removing substantially all the lipid droplets from the cells of the embryo, followed by the step of reducing the temperature of the embryo to a storage temperature suitable for storage of the embryo, said reduction of temperature being effected without lipid from said lipid droplets being redistributed into the embryo to an extent that significant damage is occasioned to the embryo during the reduction in temperature.

4. The method as in claim 3 wherein the lipid droplets are removed from the cells into a perivitelline space of the embryo and the temperature is reduced at a rate sufficient to prevent the redistribution of lipid back into the cells of the embryo.

5. The method as in claim 3 wherein step of removing substantially all the lipid droplets is achieved by polarizing the lipid droplets of the embryo, and the method further includes the step of physical removal of the lipid droplets from the embryo.

6. The method as in claim 5 wherein the step of physical removal includes piercing the outer surface of a cell or cells of the embryo with a micropipette, contacting the lipid with the pointed tube and applying suction to the lipid to remove lipid through the micropipette.

7. The method as in claim 6 wherein the cells of the embryo are treated with an cytoskeletal inhibitor, to thereby minimise damage caused by piercing the cells.

8. The method as in claim 7 wherein the inhibitor is selected from the group comprising cytochalashin and Demecolcine.

9. The method as in claim 8 including the further step of *in vitro* culturing the embryos after physically removing the lipid droplets before the step of reducing the temperature.

10. The method as in claim 3 wherein the step of removing substantially all the lipid droplets from the cells of the embryo is achieved by *in vitro* culturing of said embryo.

11. The method as in either claim 1, 2 or 3 wherein the temperature is reduced at a stage of perihatching or earlier.

12. The method as in claim 11 wherein the temperature is reduced at a stage earlier than perihatching.

13. The method as in claim 12 wherein the temperature is reduced at the single cell stage.

14. An embryo which is cryopreserved using the method of claim 1.

15. A method of implantation of an embryo, comprising the step of cryopreserving a porcine embryo as in claim 1, thawing the embryo and implanting the embryo into a recipient.

16. A method of producing an animal using cryopreserved embryos of claim 11, which embryos are then thawed and implanted into recipients and allowed to gestate to give rise to said animal.

17. An animal produced by a method according to claim 16.

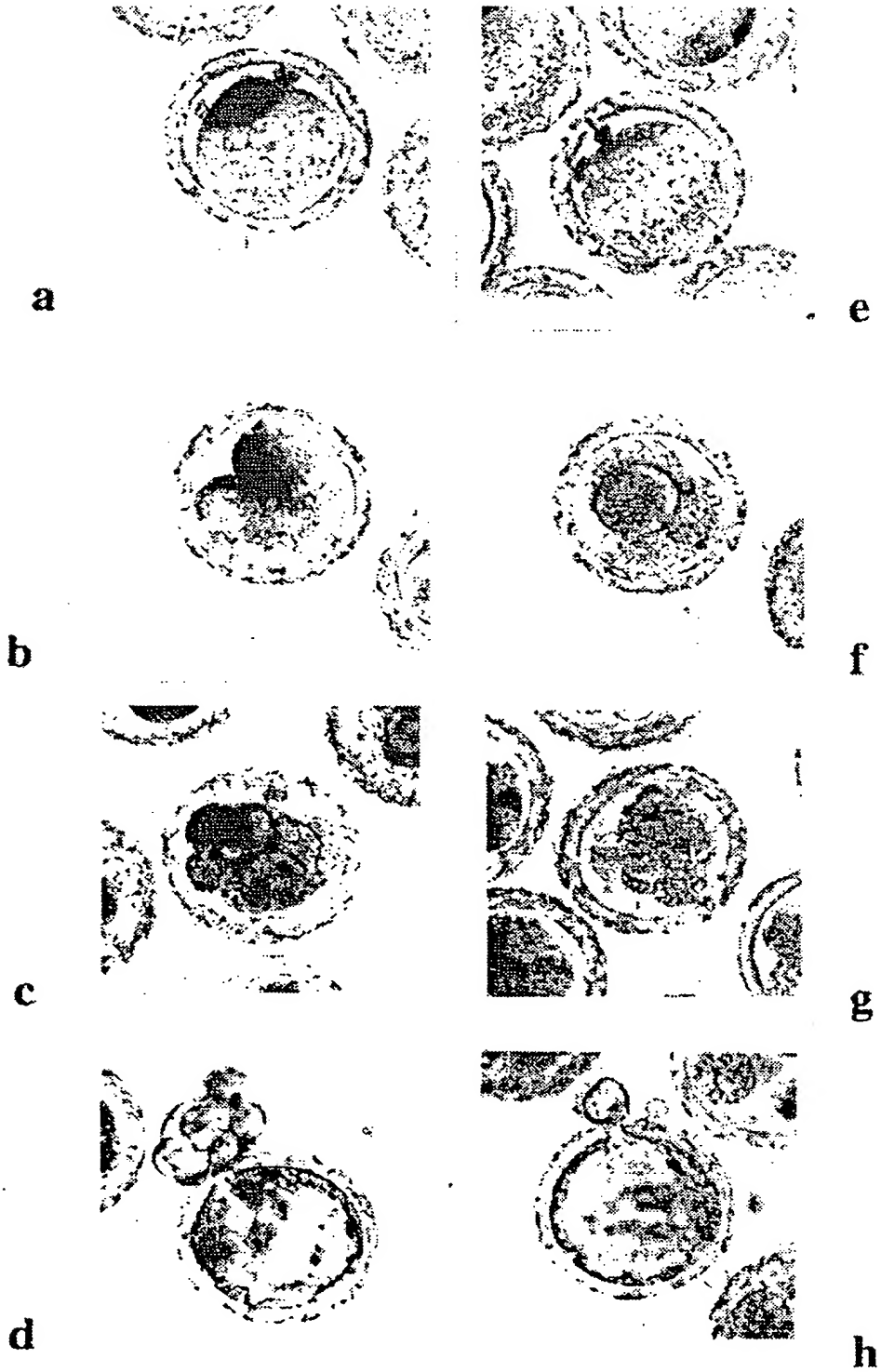

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00474

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[6] A01N 1/00, A01N 1/02, A01K 067/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: A01N 1/00, 1/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
BIOT, JAPIO, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	GB,A,2219923 (INSTITUT PROBLEM KRIOBIOLOGIL I KRIOMEDITSINY AKADEMIL NAUK UKRAIN SKOL SSR) 28 December 1989 (28.12.89) whole document	1-17
A	AU,A,73995/74 (AUGSPURGER) 8 April 1976 (08.04.76) page 35 line 1 to page 36 line 16	1-17
A	Patent Abstracts of Japan, C-740, page 100, JP,A,2-117601 (ITOCHU SHIRYO K.K.) 2 May 1990 (02.05.90)	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

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27 October 1994 (27.10.94)

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8 Nov 1994 (8.11.94)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
A	Patent Abstracts of Japan, C-510, page 163, JP,A,63-35501 (SNOW BRAND MILK PRODUCTS) 16 February 1988 (16.02.88)	1-17

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
GB 2219923						
JP 63035501						
AU 73995/74	BE 822358	BR 7409179	DE 2454674			
	FR 2569105	GB 1486468	IN 140864			
	IN 148179	JP 50157180	JP 60033506			
	JP 60246751	NL 7415307	NZ 175999			
	US 3854470	US 3866598	US 3906929			
JP 2117601	JP 6051601					

END OF ANNEX